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13. Abstract Understanding how mutations arise in non-growing cells will help illuminate mechanisms of oncogenesis, tumor progression, and resistance to chemotherapeutic drugs. In this project, I have been addressing how antibiotic resistance mutations occur in non-, or slowly-growing enterobacteria cells. Previously, our laboratory used a plasmid-assisted system to discover that RecA (an hRAD51 homolog) and RecBCD recombination repair proteins are necessary for the acquisition of ß-lactam drug-resistant mutations in the *Escherichia coli* chromosome during stationary-phase. The data suggest that the SOS DNA damage-repair response, recombinational DNA repair, or both, are involved in the mutation pathway. I have improved upon the original *E. coli* plasmid-based system to further examine the genetic and biochemical processes involved in this mutational mechanism.. Initial results in this improved system suggest that ß-lactam resistance mutations occur not only in a growth-dependent manner but also in response to stress induced by starvation. The starvation conditions may mimic the environment pathogens or tumor cells may encounter in inflicted patients under stress, chemotherapeutic treatment, or other anti-tumor drug regimen in which cells are in a state of slow-, or non-growth. In addition, I have engineered a reporter construct that will allow the enrichment of those cells undergoing stationary-phase mutation so the genetic and biochemical intermediates involved in this mutation mechanism may be studied.

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Table of Contents

Introduction

When cancers develop in tissues of non-dividing or slowly growing cells, the first cancerous cell must acquire mutations to become transformed and escape growth regulation. Mutational mechanisms unique to non-growing cells may facilitate this progression from quiescence to division. Similarly, when tumors develop resistance to chemotherapeutic drugs, such as mitotic inhibitors, they acquire mutations and other changes that allow growth in the presence of the drugs. Moreover, the growth stage when mutation is most active is not known. G_0, the analog of bacterial stationary phase, could be an important period for spontaneous mutation. To understand these processes, it is important to elucidate the mechanism(s) of mutation in non-dividing and slowly growing cells.

A mutation mechanism has been described in stationary-phase *Escherichia coli* that requires recombination proteins, and produces sequences different from growth-dependent mutations (18, 20). Stationary-phase mutation is best understood in the *E. coli* Lac assay in which cells with a *lac* +1 frameshift allele on an F' plasmid generate Lac^+ mutants upon starvation on lactose medium (3). The stationary-phase mutations, but not growth-dependent Lac^+ mutations, require recombination proteins (RecA, RecBCD, RuvABC) and the bacterial SOS DNA damage-repair response (6, 9, 10, 14). Furthermore, DNA mismatch-repair protein MutL is transiently limited during stationary-phase mutation (8, 13). Research from our lab determined that error-prone DNA polymerase IV (encoded by *dinB,* the ortholog of DINB1 in humans) is the SOS component required for Lac^+ stationary-phase mutation (15).

The *E. coli* recombination and DNA repair proteins important for stationary-phase mutation have human homologs implicated in breast and other types of cancer. For example, Rad51, a homolog of *E. coli* RecA, interacts with breast cancer tumor suppressor proteins BRCA1, and BRCA2 as well as the p53 tumor suppressor protein (16, 19, 21). Mutations in these proteins are associated with predisposition to breast cancer. In addition, defects in human homologs of the *E. coli* mismatch repair proteins MutS and MutL underlie hereditary nonpolyposis colorectal cancer (HNPCC).

Mutation mechanisms that occur in non-dividing or stationary-phase cells may generate ß-lactam antibiotic resistance mutations. ß-lactam antibiotics kill actively dividing bacteria, and so those cells that are not dividing have an opportunity to acquire resistance mutations *via* stationary-phase mutation. Chromosome-encoded AmpC ß-lactamases inactivate specific ß-lactam antibiotics and are widespread in enterobacteria. *E. coli* carries all of the genes required for *ampC* production except for *ampR*, the transcriptional activator of *ampC*. Loss-of-function mutations in *ampD*, an indirect regulator of *ampR*, are found in ß-lactam resistant pathogens and cause resistance in *E. coli* when the Enterobacter *ampRC* genes are expressed from a plasmid (12). The focus of this project is on the mechanisms of chromosomal *ampD* mutation in stationary-phase *E. coli* to improve the understanding of how antibiotic resistance mutations can arise in quiescent cells.

Recently, our lab demonstrated stationary-phase mutation occurring directly on the *E. coli* chromosome (1). However, some targets on the *E. coli* chromosome are less prone than others to stationary-phase mutation (18). The reasons for this observation are not clear and probably relate to the fundamental mechanism underlying stationary-phase

mutation. Many aspects of the mutation processes involved may be relevant to how oncogenic mutations occur in non-dividing cells in humans, and how tumors treated with drugs that kill dividing cells may still acquire mutations that enable the drugs to be evaded.

Body

As indicated in the Research Technical Reporting Requirements on the Department of Defense website, I have included in this final report data from the entire period covered by the awarded fellowship. It should be noted that the fellowship was discontinued three months into the third and final year so that the P.I. (I) could pursue another research position that became available on a different subject (biodefense). Data from the final three months are discussed following the review of year 2 research accomplishments.

Year 1

To study mechanisms of mutation to ß-lactam resistance in enterobacteria in response to starvation, I moved the *ampRC* genes from *Enterobacter cloacae* to the *attB* site on the *E. coli* chromosome of the strain used in the Lac mutation assay described above. Moving the *ampRC* genes to the chromosome improves upon existing plasmid-based *ampD*-mutation systems because it allows genetic dissection of possible roles of DNA metabolism proteins, many of which affect plasmid stability. Mutant alleles of different DNA damage-repair and recombination genes were then added to examine the genetic requirements of ß-lactam resistance mutations on the *E. coli* chromosome.

Fluctuation tests showed that *ampD* growth-dependent mutation rates, measured by the number of ampicillin resistant mutants arising in a given population, are not affected by mutations in recombination or SOS genes. This result was anticipated as most growth-dependent mutations are caused by DNA polymerase errors during replication and do not require recombination or SOS genes. These fluctuation tests showed that our improved system for studying ß-lactam resistance mutations in *E.coli* works.

I constructed a plasmid containing a copy of the *E. coli ampD* gene to perform a complementation analysis on growth-dependent ß-lactam resistant mutants from recombination-proficient (*rec*$^+$) and *recA* cells. When the *ampD*-expressing plasmid was introduced into the growth-dependent ß-lactam resistant mutants, all clones lost their ampicillin resistant phenotype, showing that the growth-dependent mutations in these strains were in *ampD*. Sequence analysis of 20 mutants from the *rec*$^+$ and *recA* cells further showed that, as expected, there was no difference in the growth-dependent mutation patterns between recombination proficient and deficient strains.

I had not been able to reproduce previous results in which *ampD* mutants arose over time in stationary-phase *E. coli* under ß-lactam selective pressure. However, I did discover an equally important phenomenon in that *E. coli* harboring the *ampRC* genes acquires *ampD* mutations over time in stationary-phase cells starved on lactose. The accumulation of *ampD* mutants requires RecA, and is promoted at a greater frequency in RecG-deficient cells, similar to Lac$^+$ stationary-phase mutants. These initial

observations show that the growth-dependent mutation mechanism is not responsible for the late-arising mutants and that a stationary-phase mutation mechanism similar to that seen in the Lac system may be responsible for these mutations on the chromosome. Further experiments using mutant alleles of other recombination and SOS genes will be performed to determine whether this is indeed the case.

Evidence from the Lac system shows that stationary-phase mutation occurs in a subpopulation of cells that undergoes transient hypermutation during starvation (18, 20). Additional studies will determine whether the ampicillin resistant mutants also arise from a hypermutating subpopulation. The defining characteristics of the hypermutable subpopulation of cells will contribute greatly to our understanding of stationary-phase mutation. For example, answering the question of what is the signal, or signals, that initiates hypermutation will give further insight as to the origin of mutation in a slowly-growing cell. To answer such questions I plan to isolate, or enrich for, the hypermutable subpopulation so that their defining feature(s) can be studied with biochemical methods.

To aid in the purification of the hypermutable subpopulation, a reporter construct was generated that incorporates the SOS requirement for stationary-phase mutation. The Green Fluorescent Protein (GFP) gene, *gfp*-m2, was inserted into a plasmid under the control of the *sulA*, SOS-inducible promoter to identify cells induced for the SOS response. SulA is a protein that inhibits cell-division in the presence of DNA damage during the SOS response and, during normal cell growth, is repressed by the LexA SOS-repressor protein. This *sulA*-promoter/*gfp* construct was incorporated into the *E. coli* chromosome to ensure single-copy levels of *gfp* expression. Using fluorescence microscopy, I have shown that cells lacking the LexA protein express GFP at high quantities. In cells expressing wild-type LexA protein, the number of cells that are *gfp*-induced is reduced approximately 1,000-fold. This result shows that *gfp* expression is under LexA SOS-repressor control, and that the construct is functioning as designed. In future experiments, starving, stationary-phase cells will be purified by flow cytometry based on their SOS-induction phenotype and tested for hypermutability to Lac^+ as well as resistance to ß-lactams. It is expected that the sorted cells will demonstrate hypermutability by showing a greater mutation frequency per cell compared to the cells that were sorted against. DNA and proteins can be purified from the sorted cells and will be compared to the DNA and proteins from control cells to identify the initiating signals responsible for stationary-phase mutation.

Year 1 Key Research Accomplishments (items 1-5 published in *Antimicrob. Agents Chemother.* 46:1535-9: Appendix 1):

- The *ampRC* genes were integrated into the *E. coli* chromosome.
- Mutant alleles of the *recA, recB, recG, lexA3, sulA, sulA lexA51,* and *dinB* recombination and SOS genes were incorporated into *ampRC*-containing cells.
- Growth-dependent and stationary-phase mutation were quantified using this system.
- Stationary-phase, but not growth-dependent, mutation requires *recA* and increases in *recG*-deficient cells.
- 20 out of 20 growth-dependent Amp^R mutations in rec^+ and *recA* cells occurred in *ampD* as determined by plasmid complementation and sequence analysis.

- A *sulA* promoter-*gfp* fusion was constructed and integrated into the *E.coli* chromosome to isolate SOS-induced cells.
- The *sulA* promoter-*gfp* fusion was demonstrated to be LexA-regulated as intended.

Response to first year summary technical issues:

The reviewer of the year one summary made several interesting and insightful comments that are addressed here.

Technical issues:

a) The reviewer commented on an apparent contradiction in mutation results reported in the *recA* background.--Fluctuation tests were done in RecA$^-$ cells to show that recombination functions are not required for growth-dependent, spontaneous mutations in *ampD*. Spontaneous mutations are traditionally related to strand-slippage events and polymerase-errors and are not perceived to require recombination functions, such as those encoded by *recA*. These fluctuation tests were performed partly as proof-in-principle experiments for the chromosomal *ampRC* system, as well as to confirm our presumption that little or no recombination-dependent spontaneous mutation is occurring in these cells.

The other mutation studies demonstrating *recA* dependence relate solely to mutation happening in stationary-phase cells after they are starved in the presence of lactose. In studies performed to date by our lab and others, mutation in stationary-phase cells is strikingly different to mutation in growing cells. One of the fundamental mechanistic differences is the requirement for recombination functions in stationary-phase, but not spontaneous (i.e. growth-dependent), mutation .

b) The reviewer questioned how roles for the RecA and RecG proteins in stationary-phase mutation are assigned, as well as how will we determine which function(s) of the multi-purpose RecA protein is important for stationary-phase mutation.--Various genetic requirements for stationary-phase mutation were determined in experiments where strains isogenic to the parent *ampRC*$^+$ *E. coli* were deleted for genes of interest (e.g. *recA* and *recG*). It was found that *recA* is absolutely required for *ampD* stationary-phase mutation, and deletion of the *recG* gene leads to a greater frequency of *ampD* mutation. As a result, *recA* is believed to be a primary component of the stationary-phase mutation mechanism, while the role of *recG* is not clear.

As the reviewer points out, RecA is also a critical component of the bacterial SOS DNA damage-repair response. To discern which functions of RecA are required for stationary-phase mutation, genes specific to RecA-mediated DNA recombination/repair and genes belonging to the SOS response were deleted in isogenic *ampRC*$^+$ strains and tested for their ability to undergo stationary-phase *ampD* mutation. It was found that both recombination and the SOS-induced *dinB* gene are required for mutation in this system (described below in year 2 results). (DinB, or DNA polymerase IV, is an error-prone DNA polymerase which has homologs in all three domains of life [archaea, bacteria, and eukaryotes—including humans].) Therefore, it appears both the SOS

inducing- and recombination functions of RecA are required for stationary-phase *ampD* mutation.

c) The reviewer brought up complications with working with a starvation model for studying stationary-phase mutation, particularly the presence of free radicals, which may promote stress.--The role of damaging radicals and DNA base-analogs which may be present in higher quantities during starvation and/or stationary phase is of great interest to our laboratory in both the context of the original Lac system as well as our *ampRC* system and, of course, in eukaryotic cells. Preliminary Lac stationary-phase mutation studies have been initiated (by others in our lab) with strains deleted for genes involved in the repair of oxidative damage in the cell. These studies have generated potentially interesting results that need to be pursued in both the Lac and *ampRC* systems. The relevance to breast/eukaryotic cancer systems will be explored as well.

Year 2

The genetic requirements for ß-lactam resistance mutation was studied further in stationary phase cells starving on lactose. Among other interesting results, I found that DNA PolIV is required for stationary-phase ampicillin resistance mutation as it was found to be for Lac stationary-phase mutation. When the *dinB* gene is interrupted by a kanamycin-resistance gene cassette, mutation to ampicillin resistance drops to background levels in cells starving on lactose. Recent papers suggest the possible involvement of three of the four DinB (PolIV)/UmuDC superfamily polymerases present in vertebrates in somatic hypermutation of the immunoglobulin genes (4, 17, 22).

I have also found that stationary-phase ß-lactam resistance mutation requires the DNA recombination protein *recB* which, when mutated, eliminates the RecBCD double-stranded DNA (dsDNA) helicase/exonuclease. RecBCD normally prepares DNA for recombination by unwinding dsDNA and loading RecA protein onto the newly created ssDNA to prepare it for recombination (5). The requirement of RecBCD suggests that DNA double-strand ends (DSEs) are an intermediate in the stationary-phase mutation mechanism. How these DSEs are generated is of great interest and is currently under investigation. Damage from free radicals, as mentioned by the reviewer from my year one report, is one hypothesis that we are testing.

In year 1, I analyzed growth-dependent *ampD* mutant sequences to ascertain the sequence spectrum of growth-dependent mutation in our system (Appendix 1). In year two, I began sequencing *ampD* mutants that were isolated from our stationary-phase mutation experiments to compare the sequence spectra of growth-dependent and stationary-phase mutation in our system. So far, I have collected 20 *ampD* stationary-phase mutant sequences and have found that the sequence spectrum appears significantly different in stationary-phase, compared to that of growth-dependent, mutants. Hotspots (where mutations seem to cluster) in the growth-dependent mutants, were non-existent in the stationary-phase mutants. Also, there is a high proportion of substitution mutations in the stationary-phase mutants compared to the growth-dependent mutants. Among the substitution mutations, I found a high-proportion of G to T transversions. Interestingly, G-C to T-A transversions were found to be increased nearly 100-fold when PolIV is overexpressed in *E. coli* (11). Also, G to T transversions are found in cells unable to

repair 8-oxyguanine oxidative damage. This sequence information therefore points to/supports two potential pathways leading to mutation: one through PolIV (already shown through genetic requirement testing), and one possibly through oxidative damage. The role of oxidative damage in the stationary-phase mutation pathway can be tested by deleting genes required for the repair of 8-oxyguanine damage (e.g. *mutY*—such experiments are underway in our laboratory), however the results of such experiments have to be carefully interpreted and followed up, because as the reviewer from year 1 mentioned, primary and secondary causes and effects of this mutation process will be detected in our system.

In year 1, I reported on a *sulA* promoter/*gfp* construct that will enable us to enrich for starving, stationary-phase cells undergoing an SOS response from cells that are not at different times during experimental trials. It is expected that the sorted cells that are green will demonstrate hypermutability to ß-lactam resistance and to Lac^+ compared to cells sorted against. The DNA and proteins in this subpopulation can be purified to further examine the changes and signals occurring in these cells during stationary-phase mutation. Over the past year, I performed several follow-up experiments with these cells to help me ascertain whether future sorting experiments will be successful. In addition to the original *sulA* promoter/*gfp* construct, I have made recombination protein-deficient derivatives as controls and additional strains to test in our sorting experiments. These cells were examined by fluorescence microscopy to determine their level of SOS induction at the cellular level. It was found that certain recombination-deficient backgrounds have a higher fraction of cells induced for the SOS response compared to the recombination-proficient parent strain. However, never were all cells chronically SOS-induced. These observations suggest that our construct will enable us to sort subpopulations of SOS-induced cells from those not experiencing an SOS response.

Year 2 Key Research Accomplishments:

- Additional alleles have been introduced into the *ampRC*$^+$ tester strain including *recB* and *ruvC*
- ß-lactam resistance mutation was shown to be PolIV, RuvC (Holliday Junction resolvase), and RecB dependent
- Sequence spectra of stationary-phase mutants shows a different mutation pattern compared to growth-dependent mutants (supports PolIV requirement and suggests possible role of oxidative damage in stationary-phase mutation)
- *gfp*- fusion studies show discrete levels of SOS induction within bacterial populations
- Control experiments leading to the publication of our stationary-phase mutation work are underway
- Paper describing the *ampRC*$^+$ system has been published (*Antimicrob. Agents Chemother.* 46:1535-9: Appendix 1)

Year 3 (3 months)

Initial studies were performed to test whether the stationary-phase resistant mutants isolated in our experiments arise from a hypermutable subpopulation, as has been shown in some of the other stationary-phase mutation systems (e.g. 2). Over 2,000

growth-dependent and stationary-phase, ampicillin-resistant mutants were isolated from our mutation assays and screened for mutations at other loci (i.e. secondary mutations—indicative of hypermutation). No difference was observed in the frequency of secondary mutations found in growth-dependent, ampicillin-resistant mutants and stationary-phase, ampicillin-resistant mutants, suggesting that, unlike other stationary-phase mutation systems examined in our laboratory, hypermutation may not be a characteristic of stationary-phase *ampD* mutation. However, importantly, the number of mutants screened thus far is relatively low for significant statistical comparisons to be made. Hypermutation experiments need to proceed further in order to determine if the initial trend is valid.

An additional experimental control, which can be performed in conjunction with the hypermutation experiments, needs to be completed to show that the ampicillin-resistant mutants arising in stationary-phase are not heritable mutators. It has been demonstrated that, in a given bacterial population, there is often a small percentage of cells that are mutator (capable of mutation at higher frequencies than the rest of the bacterial population—often the result of a defect in mismatch repair)(7). Experiments have shown that mutants isolated in other stationary-phase mutation assay systems are not simply mismatch-repair defective—an important control that shows stationary-phase mutation to be independent of mutation in growing or mismatch-repair defective cells. For our purposes, ampicillin-resistant mutants that phenotypically exhibit mutation at another loci (e.g. unable to metabolize maltose, as indicated when screened on a MacKonkey-maltose agar plate) can be tested to see whether these isolates accumulate mutations at a rate associated with mismatch-repair-deficient strains of *E. coli*. The few ampicillin-resistant mutants screened thus far suggest that the ampicillin resistant mutants that arise in the stationary-phase mutation experiments are not mutators. As with the hypermutation experiments, more ampicillin-resistant mutants need to be screened for the results to be statistically significant.

Fluorescence microscopy experiments using the *sulA-gfp* reporter system (described in the Year 1 and 2 results) have shown that approximately 0.5 to 2.0% of wild-type *E. coli* cells are SOS-induced (as defined by induction of the *sulA* promoter) in a stationary-phase population. This suggests that there are DNA lesions in approximately 2% of the bacterial cells in a given non-dividing population that require repair before cell-division can resume. In cells where the RecG Holliday junction-migration protein is absent, the proportion of cells that are SOS-induced is approximately 5-10%. RecG has been shown to be needed for DNA replication restart, in some circumstances, when the DNA replication fork encounters DNA lesions. It has been predicted that DNA damage persists in cells deficient in RecG, and that level of persistence, which leads to an SOS response, can now be quantified using this construct. A parallel set of experiments (not mentioned in the original **Statement of Work**) can further be performed with this reporter construct to compare the levels of DNA damage in various genetic backgrounds to ascertain the relative importance of various proteins in DNA damage-repair.

Further experiments using the reporter construct have been ongoing in the Rosenberg laboratory following my departure. These experiments have begun to address the questions/aims that were proposed for the construct in the original **Statement of Work**. SOS-induced bacterial cells have been successfully sorted using flow-cytometry. Therefore, potentially hypermutating cells can now be isolated from stationary-phase

populations based on SOS-induction. These sorted cells can be further examined for chromosomal breakage, mismatch repair protein depletion, and differential gene expression as outlined in the original **Statement of Work**.

Year 3 Key Research Accomplishments:

- Hypermutation experiments have begun in the ampicillin-resistance chromosomal mutation system. Initial results show that hypermutation may not be as prevalent as in the other stationary-phase mutation systems examined in the Rosenberg laboratory.
- Initial heritable mutator control experiments suggest that the stationary-phase *ampD* mutations are not simply mutations in mismatch repair-deficient cells.
- Experiments using the *sulA-gfp* reporter show promise with the ability to sort potentially hypermutating cells from stationary-phase populations for the purpose of isolating the signal(s)/precursor(s) to the observed mutation pathway(s) in non-dividing cells.

Reportable Outcomes

Abstract submissions:

2002 Gordon Research Conference on Mutagenesis. July 28-August 2, Lewiston, Maine. *E. coli* DNA PolIV/DinB and recombination proteins are required for stress-induced antibiotic resistance mutations.

2002 Era of Hope 2002 Department of Defense Breast Cancer Research Program Meeting, September 25-September 28, Orlando, Florida. Recombination proteins and the PolK ortholog, PolIV are required for stationary-phase antibiotic resistance mutation.

2001 Molecular Genetics of Bacteria and Phages Meeting, July 31-August 5, University of Wisconsin, Madison. Resistance to ß-lactam antibiotics mediated by stationary-phase mutation.

Publications:

Petrosino J.F., Pendleton A.R., Weiner J.H., Rosenberg S.M. 2002. Chromosomal system for studying AmpC-mediated beta-lactam resistance mutation in Escherichia coli. Antimicrob Agents Chemother. **46**:1535-9.

Petrosino, J. F., Hastings, P. J., Rosenberg, S. M. (2001). RecBCD enzyme, pathway. Encyclopedia of Genetics. Sydney Brenner and Jeffrey H. Miller, Editors-in-chief. Academic Press, N.Y.

Conclusions

The genetic requirements observed for stationary-phase ß-lactam resistance mutation so far suggest that a similar mechanism underlies both the *ampRC*$^+$ and the Lac

systems. However, *ampD* mutation takes place on the bacterial chromosome and not on the F' and differences between mutation in these two regions is currently being investigated. The *ampRC*$^+$ system provides a first look at the full sequence spectra of recombination/PolIV-dependent stationary-phase mutation. The F' plasmid-based Lac and chromosomal Tet^R systems specifically select for –1 frameshift mutations and therefore display a strong bias when looking at their respective sequence patterns. Data thus far suggest that *ampD* stationary-phase mutations are significantly different from *ampD* growth-dependent, spontaneous mutations. The data supports the role of PolIV as the polymerase generating the mutations, and also hints at the role of oxidative damage as a potential component of the mutational mechanism as well.

Preliminary experiments leading to flow-cytometry sorting of the hypermutable cell subpopulation show that DNA damage is constantly occurring in a fraction of a given cell population and that this fraction is different depending on the genetic background of the cells being observed. Actual sorting of the cells shows great promise and will not only generate more reliable numbers as far as the size of various SOS-induced populations, but will also serve as a means to isolate the hypermutable subpopulation and identify the factors that lead to mutation in non-dividing cells.

So What...

When cancers develop in tissues of non-dividing or slowly growing cells, the first cancerous cell presumably must acquire mutations to become transformed and escape growth regulation. Similarly, when tumors develop resistance to chemotherapeutic drugs, such as mitotic inhibitors, they acquire mutations and other changes that allow growth in the presence of the drugs. Mutational mechanisms unique to non-growing cells may facilitate both of these processes. G_0, the analog of bacterial stationary phase, could be an important period for spontaneous mutation. The goal of the work performed here, and which has continued following my departure from the Rosenberg laboratory, is to understand how mutations occur in non-dividing cells using the well-established genetic system of *E. coli*. Several DNA repair and recombination proteins, including RecA and DinB, which have human homologs, have been implicated in stationary-phase mutation of *E. coli*. The AmpD system developed here has enabled further study of stationary-phase mutation on the bacterial chromosome instead of the F' sex-plasmid which may or may not acquire stationary-phase mutations using the same mutational mechanism(s). To date, chromosomal and F' stationary-phase mutation show the same genetic requirements for DNA recombination and repair proteins, suggesting that the processes at both loci are similar if not identical. The chromosomal AmpD system also has made it possible to study the complete sequence spectrum of stationary-phase mutation, presumably without bias for certain types of mutations (e.g. frameshifts). The stationary-phase mutation spectrum from the AmpD system has provided initial evidence that suggests oxidative damage may play an important role in mutation in non-dividing cells. Other experiments in the Rosenberg laboratory are directed at examining this possibility.

It was previously determined that the ability to mount an SOS-response to DNA damage is required for stationary-phase mutation. Therefore, the *sulA-gfp* reporter system may potentially enable the direct identification of the substrates or intermediates that lead to stationary-phase mutation by allowing for the hypermutable subpopulation

from the F' system to be separated from those cells not undergoing hypermutation. (The reporter construct is currently located at the same chromosomal locus, *attB*, as the *ampRC* genes required for the AmpD system, therefore the *sulA-gfp* construct cannot be used in the AmpD system, at least not as currently constructed.) Flow cytometry has already been used to isolate cells undergoing an SOS response from those that are not. The next step is to sort and study those cells that have been starving in stationary-phase to try and isolate the factor(s) that underlie stationary-phase mutation.

Together, the chromosomal AmpD stationary-phase mutation system, and the *sulA-gfp* reporter construct have enabled, and will continue to enable, study of the basic mechanisms and causes of mutation in non-dividing cells in a tractable model organism for DNA damage and repair. This knowledge should aid in the understanding of origin of mutations in non-dividing cells of higher organisms.

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NOTES

Chromosomal System for Studying AmpC-Mediated β-Lactam Resistance Mutation in *Escherichia coli*

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In some enterobacterial pathogens, but not in *Escherichia coli*, loss-of-function mutations in the *ampD* gene are a common route to β-lactam antibiotic resistance. We constructed an assay system for studying mechanism(s) of enterobacterial *ampD* mutation using the well-developed genetics of *E. coli*. We integrated the *Enterobacter ampRC* genes into the *E. coli* chromosome. These cells acquire spontaneous recombination- and SOS response-independent β-lactam resistance mutations in *ampD*. This chromosomal system is useful for studying mutation mechanisms that promote antibiotic resistance.

Mutation is a primary cause of bacterial resistance to antibiotics. Some mutations promote resistance directly (e.g., quinolone resistance mutations in genes encoding its *Escherichia coli* targets, *gyrA* and *gyrB* [16]). Other chromosomal mutations can cause mutator phenotypes that increase the likelihood of acquiring a resistance mutation (42). Mutations also ameliorate the otherwise deleterious effects on cell growth and physiology of some antibiotic resistance-conferring mutations (22). Although antibiotic resistance has been studied intensively, the mechanisms that generate resistance mutations are poorly understood.

In addition to spontaneous mutation in exponentially growing cells (growth-dependent mutation), other mutation mechanisms exist that may pertain to antibiotic resistance (34, 36, 37). For example, factors such as antibiotic concentration (23), environmental conditions (12), or other stress-inducing phenomena (1, 34, 36) may enhance or repress mutational machinery that leads to resistance mutations (for a review, see reference 31). Some mutation mechanisms or factors may be more important when the organism is under suboptimal growth conditions, as is probably the case during certain stages of an infection. In this study, we utilize a relatively well-described β-lactam resistance pathway as a model system to begin dissecting the mechanism(s) of antibiotic resistance mutation using the tools of *E. coli* genetics.

Chromosomally encoded AmpC β-lactamases confer β-lactam antibiotic resistance in many pathogenic and opportunistic bacteria and are ubiquitous in enterobacteria, except for the salmonellae, klebsiellae, *Proteus mirabilis*, *Shigella flexneri*, and *Shigella dysenteriae* (30, 32). Their expression is inducible in all but *E. coli* and the shigellae (30). In inducible strains, *ampC* transcription is controlled by the transcriptional activator AmpR (2). AmpR activity is regulated allosterically by two cell wall components, 1,6-anhydromuropeptide and UDP-*N*-acetylmuramic acid-pentapeptide (UDP-MurNAc-pentapeptide). The first allows, and the second blocks, AmpR transcriptional activator activity at *ampC* (19). AmpD converts (activator-promoting) 1,6-anhydromuropeptide to (activator-blocking) UDP-MurNAc-pentapeptide, which then binds AmpR and blocks *ampC* transcription. Thus, loss-of-function mutations in *ampD* cause 1,6-anhydromuropeptide accumulation and constitutively induced AmpC β-lactamase production (7, 20, 21, 27). *ampD* missense and nonsense mutations are common in AmpC-mediated, β-lactam-resistant clinical isolates (25, 38). Also, some β-lactam antibiotics can induce expression of *ampC* by causing an increase in the cytoplasmic concentration of 1,6-anhydromuropeptide (7, 30).

E. coli lacks *ampR*, and low-level *ampC* expression results from a promoter embedded in the overlapping fumarate reductase (*frdABCD*) operon (13). High-level β-lactam resistance, mediated by *ampD* loss-of-function mutation, can be reconstituted in *E. coli* when the *ampR* and *ampC* genes of other enterobacteria are expressed from a plasmid (28, 35). We have integrated the *ampRC* genes from *Enterobacter cloacae* into the *E. coli* chromosome to assay *ampD* mutation, as selected by its β-lactam resistance phenotype. Background resistance imparted by the native *ampC* gene does not interfere with assays involving the reconstituted system. Integrating the *ampRC* genes into the chromosome improves upon previous plasmid-based *ampRC* expression systems by allowing genetic analyses not possible previously, first, because many mutant alleles used to study DNA repair, recombination, and mutation cause plasmid instability (e.g., reference 6). Second, the single-copy *ampRC* locus more closely models the situation in clini-

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TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Relevant genotype	Reference or source
Strains		
DM49	*lexA3*	29
FC526	Δ*recG263*::*kan*	11
GY8322	Δ(*srlR-recA*)*306*::Tn*10*	S. Sommer; ENZ280 (8) carrying the K5353 mini-F plasmid (9)
SMR821	*lexA3 malB*::Tn*9*	33
SMR1827	FC40 *sulA211*	33
SMR4562[a]	*rec*$^+$*att*λ$^+$	33; genotype identical to FC40 (4)
SMR4649	FC40 *sulA211 lexA51*	33
SMR5078	*recB21 recC22 sbcB15 sbcC201* $hsdr_K^-m_K^+$ (λ*xis1 cIts857*)	14
SMR5156	SMR4562 (λ*xis1 cIts857*)	SMR4562 × λSR446 (14)
SMR5201	*recB21 recC22 sbcB15 sbcC201* $hsdrK^-mK^+$ Δ*att*λ::*ampRC*	SMR5078 × DNA from pJP2
SMR5222	SMR4562 Δ*att*λ::*ampRC*	SMR 5156 × P1 SMR5201
SMR5225	SMR5222 Δ(*srlR-recA*)*306*::Tn*10*	SMR5222 × P1 GY8322
SMR5578	SMR5222 Δ*recG263*::*kan*	SMR5222 × P1 FC526
SMR5652	SMR5222 Δ*recG263*::*kan* Δ(*srlR-recA*)*306*::Tn*10*	SMR5578 × P1 GY8322
SMR5701	SMR1827 (λ*xis1 cIts857*)	SMR1827 × λSR446
SMR5702	SMR4649 (λ*xis1 cIts857*)	SMR4649 × λSR446
SMR5715	SMR5222 *lexA3 malB*::Tn*9*	SMR5222 × P1 SMR821
SMR5725	SMR5222 *sulA211 lexA51*	SMR5702 × P1 SMR5201
SMR5749	SMR5222 *sulA211*	SMR5701 × P1 SMR5201
Plasmids		
pEc1c	*E. cloacae ampRC*$^+$	35
pJP2	pTGV-light *ampRC*$^+$	This work
pJP19	pACYC184 *ampD*$^+$	This work[b]

[a] Full genotype is Δ(*lac-pro*)XIII *thi ara* Rif^r [F′ α45, *lacI*^{q} *lacI33*Ω*lacZ*].

[b] Plasmid pJP19, carrying the *E. coli ampD*$^+$ gene and promoter, was created by amplifying *ampD*$^+$ from *E. coli* wild-type strain MG1655 (3) chromosomal DNA using primers AmpD no. 1, 5′-GGGTTTTCATGAGAGGCGGCATGT TAAAACTCCAG-3′; and AmpD no. 2, 5′-GGGTTTAAGCTTTCATGTTGT CTCCTTGCTGACCAG-3′. The primers incorporate terminal *Bsp*HI and *Hin*dIII restriction sites at the 5′ and 3′ ends (respectively) of the amplified fragment. Amplified *ampD*$^+$ DNA and pACYC184 DNA (5) were digested with *Bsp*HI and *Hind*III, and the *ampD*$^+$ fragment was ligated into pACYC184. pJP19-mediated *ampD* expression was confirmed by complementation to β-lactam sensitivity of four independent *ampD* β-lactam-resistant mutants.

cally relevant enterobacterial resistance (30). Third, this chromosomal system excludes mutations that confer β-lactam resistance by increasing plasmid (and, therefore, *ampC*) copy number.

An *ampRC* expression cassette in the *E. coli* chromosome was constructed as follows. The strains and plasmids used are shown in Table 1. SMR5222, an *E. coli* strain carrying the *E. cloacae ampRC*$^+$ genes in the *E. coli* chromosome, was constructed using the TGV (transgenic vector) system for integrating linear DNA cassettes into the *E. coli* chromosome (14). *E. cloacae* strain MHN1 *ampRC*$^+$ genes were isolated from plasmid pEc1c (35) by digestion with *Bam*HI and *Sal*I and were ligated into *Bgl*II- and *Xho*I-digested pTGV-light (14) plasmid DNA, creating pJP2. pJP2 was digested with *Nde*I to generate an *ampRC*$^+$ fragment flanked on both sides by homology to the *E. coli* attachment site for phage λ (*att*λ) for linear transformation in the TGV system (14). SMR5201, a transformant carrying *ampRC*$^+$ replacing *att*λ (confirmed by PCR as described elsewhere [14]) was used as a P1 donor to move *att*λ::*ampRC*$^+$ into SMR5156 by P1 transduction (as described elsewhere [14]) to create SMR5222. Subsequent strains are isogenic to SMR5222 and were created using standard phage P1 transduction (referenced elsewhere [14]), and the constructions are outlined in Table 1.

We used the chromosome-based *ampRC* expression system to determine rates of spontaneous (growth-dependent) ampicillin resistance (Amp^r) mutation in otherwise isogenic strains

TABLE 2. Rates of β-lactam resistance mutation in *E. coli* DNA repair-deficient mutants

Relevant genotype[a] and expt[b]	Mutation rate (mutations/cell/generation, 10^{-7})	Rate relative to *rec*$^+$ within each expt	Mean mutation rate (mutations/cell/generation, 10^{-7}) ± SE	Mean relative to *rec*$^+$ within each expt ± SE
rec$^+$				
1	0.460	1	1.4 ± 0.3	1.0
2	2.18	1		
3	2.71	1		
4	1.10	1		
5	1.60	1		
6	0.632	1		
7	1.20	1		
recA			1.0 ± 0.3	0.66 ± 0.1
1	0.242	0.53		
2	0.908	0.42		
3	2.72	1.0		
4	0.693	0.63		
5	0.950	0.59		
6	0.277	0.44		
7	1.24	1.0		
recG			2.0 ± 0.8	1.0 ± 0.2
1	0.342	0.74		
2	3.20	1.5		
3	2.38	0.88		
recA recG			1.2 ± 0.5	0.65 ± 0.1
1	0.235	0.51		
2	1.70	0.78		
3	1.79	0.66		
lexA3			0.96 ± 0.2	0.79 ± 0.1
5	1.48	0.93		
6	0.362	0.57		
7	1.05	0.88		
sulA			1.0 ± 0.1	0.93 ± 0.1
5	1.05	0.66		
6	0.665	1.1		
7	1.29	1.1		
sulA lexA5 (Def)			1.7 ± 0.3	1.5 ± 0.04
5	2.45	1.5		
6	0.895	1.4		
7	1.69	1.4		

[a] Strains used were SMR5222, *rec*$^+$ (recombination and SOS response proficient); SMR5225, *recA* (recombination and SOS response deficient [40]); SMR5578, *recG* (recombination proficient, elevated for stationary-phase-mutation [11, 15]); SMR5652, *recG recA* (recombination, SOS, and stationary-phase mutation deficient [11, 15]); SMR5715, *lexA3* (recombination proficient, SOS gene induction defective [40]); SMR5749, *sulA* (allows viability in the presence of a *lexA*-null mutation [40]); and SMR5725, *sulA lexA51*(Def) (SOS-induced genes expressed constitutively [40]).

[b] Data preceded by the same experiment number were gathered in parallel.

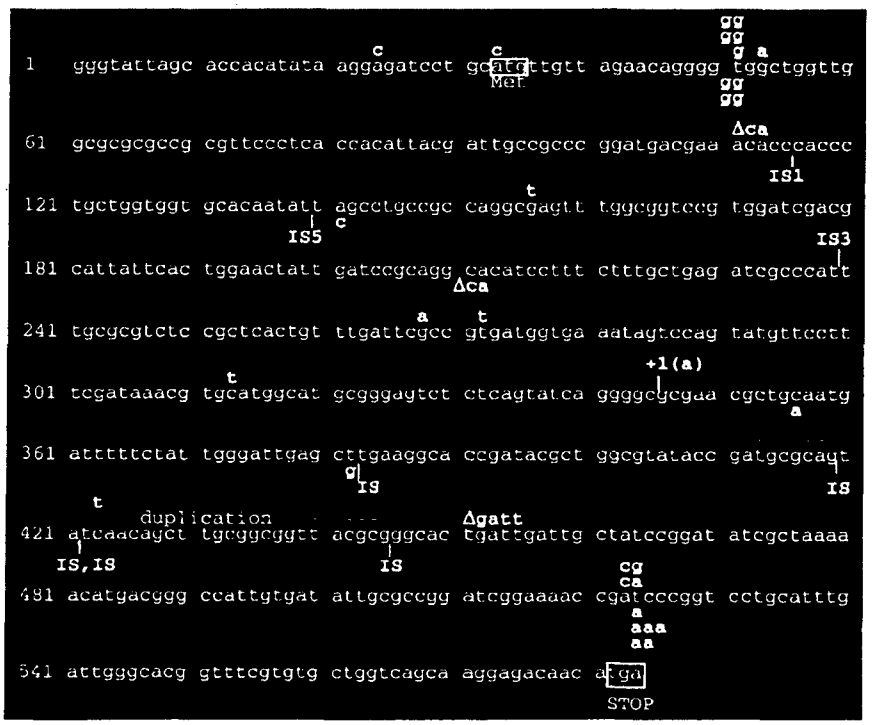

FIG. 1. Location of *ampD* mutations. Forty independent Amp^r mutants (20 from *rec*$^+$ strain SMR5222 and 20 from *recA* strain SMR5225) were screened for *ampD* mutations by complementation to β-lactam sensitivity using pJP19 (Table 1). Apparent *ampD* mutations were sequenced (Lone Star Labs, Houston, Tex.) from PCR products using primers AmpD no. 3, 5′-GCGCGTCTCCGCTCACTGTTT-3′; and AmpD no. 4, 5′-GCA TGCCATGCACGTTTATCG-3′. The PCR products were generated with primers AmpD no. 1 and AmpD no. 2 (legend to Table 1). The *E. coli ampD* sequence is given, along with 32 bp upstream of the ATG start codon. Mutations found in *rec*$^+$ mutants are indicated above, and mutations found in *recA* mutants are shown below, the sequence. IS, insertion sequence.

lacking various recombination and SOS genes. Growth-dependent mutation rates were measured in 15 tube fluctuation tests. Fifteen independent cultures for each strain were grown to saturation in 5 or 10 ml of Mueller-Hinton (MH) broth (Difco), shaking at 37°C. Cultures were diluted 10-fold, and 50 μl was plated in duplicate on MH agar plates containing 100 μg/ml ampicillin. The plates were incubated overnight at 37°C, and Amp^r colonies were counted. Viable cell counts of the saturated cultures were from dilutions plated on MH incubated overnight at 37°C. Mutation rates were calculated by the method of the median (26).

The recombination and SOS genes examined—*recA*, *recG*, *recA recG*, *lexA3*(Ind$^-$), *sulA*, and *sulA lexA*(Def)—are not required for most growth-dependent mutation; however, they are required for a mechanism of mutation observed under growth-limiting conditions of carbon starvation (for a review, see reference 37; see also references 41 and 43). Moreover, the SOS response controls several mutation-promoting proteins (40) whose possible involvement in β-lactam resistance mutation we wished to test. The β-lactam resistance mutation rate in recombination- and SOS-proficient (*rec*$^+$) cells is about 1.4×10^{-7} cell^{-1} generation^{-1} (Table 2). The strains tested displayed only small differences in growth-dependent mutation rates, indicating that recombination and SOS genes are not important for most growth-dependent β-lactam resistance mutation (Table 2).

The following experiments demonstrated that the β-lactam resistance mutations are in *ampD*. Based on prior observations in an *E. coli* model and in clinical isolates of enterobacterial pathogens (7, 27), we expected most of the β-lactam resistance mutations to be in *ampD*. To test this, 20 independent ampicillin resistant mutants (each from a separate independent culture) from the *rec*$^+$ and *recA* fluctuation test experiments were examined further. β-Lactam sensitivity was restored to each of the 40 mutants by transforming each with pJP19 (Table 1) carrying the *ampD*$^+$ gene. This complementation test shows that the ampicillin resistance mutations of the 40 independent mutants are recessive, loss-of-function mutations in *ampD*.

We determined the *ampD* sequences for each of the 40 mutants. Little difference was found between the *rec*$^+$ and *recA* backgrounds (Fig. 1), suggesting similar mutation mechanism(s) in each. Many different mutations and insertions in *ampD* conferred AmpC-mediated β-lactam resistance.

Among the eight substitution mutations identified here, two were identified previously from β-lactam resistant isolates. A Trp7Gly substitution in AmpD occurred in both the *rec*$^+$ and *recA* strains (Fig. 2) and previously in *E. coli* ampicillin-resistant mutants of cells expressing the *E. cloacae ampRC* genes from a plasmid (18). Also, we found Asp164Glu and Asp164Ala in both *rec*$^+$ and *recA*. Asp164Glu was found previously in *Citrobacter freundii* (39). Both previous *ampD* mutations were shown to cause full derepression of *ampC*, as *ampD*-null mutations do (28, 39).

Other *ampD* substitution mutations found include

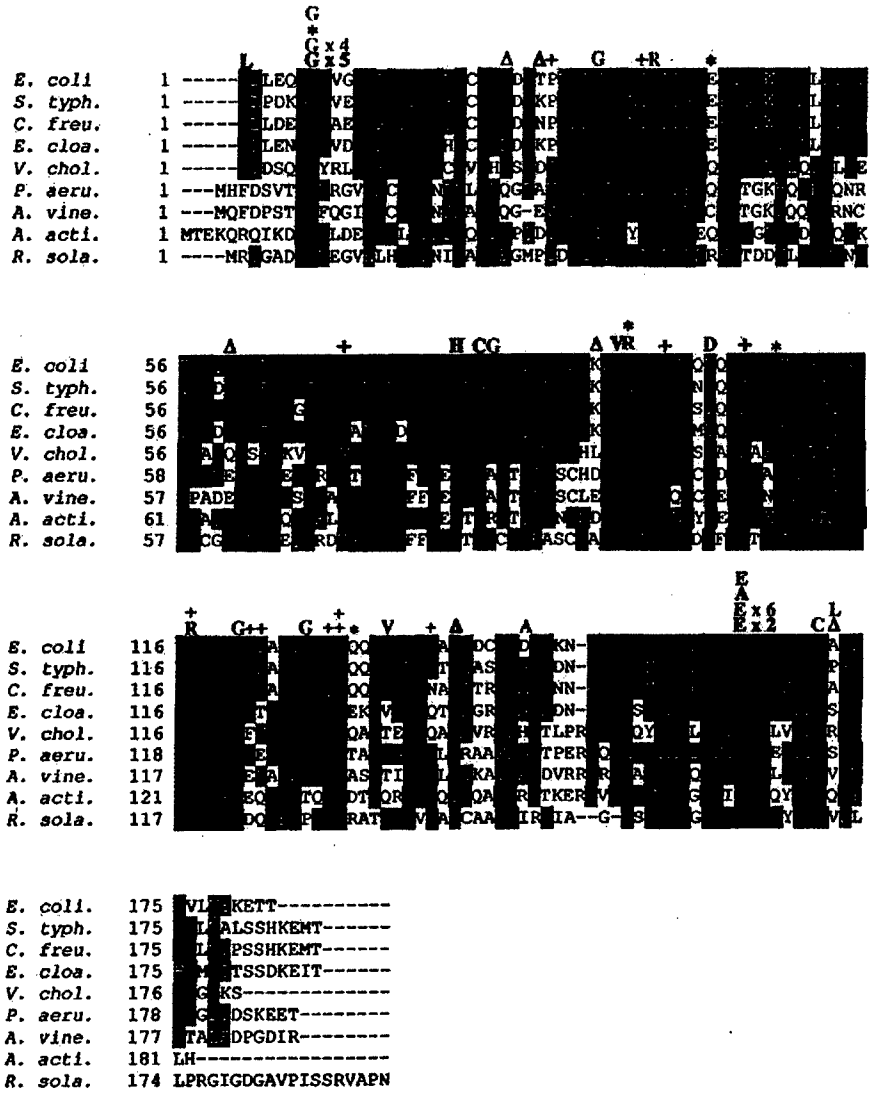

FIG. 2. Amino acid substitutions, insertions, and deletions identified in the *rec*$^+$ and *recA* mutants aligned with the *ampD* genes from nine enterobacteria. Also included are previously identified *ampD* mutant proteins from other laboratories (10, 18, 24, 28). *rec*$^+$ mutations are in black, *recA* mutations are in red, and previously identified mutations are in blue. Insertions are indicated by a plus sign (+), deletions are indicated by a delta (Δ), and nonsense mutations are indicated by an asterisk (*). Mutations isolated multiple times show the number of times that each was encountered for each strain (*rec*$^+$ or *recA*). Abbreviations: *S. typh*, *Salmonella enterica* serovar Typhimurium; *C. freu*, *C. freundii*; *E. cloa*, *E. cloacae*; *V. chol*, *Vibrio cholerae*; *P. aeru*, *Pseudomonas aeruginosa*; *A. vine*, *Azotobacter vinelandii*; *A. acti*, *Actinobacillus actinomycetemcomitans*; and *R. sola*, *Ralstonia solanacearum*. Alignment was performed using ClustalW (17) and formatted using BOXSHADE.

Ser37Arg, Arg80His, Gly82Cys, Ala94Val, and Leu117Arg (Fig. 2). Although these might or might not inactivate *ampD* fully, substitutions that alter or abolish AmpD function reveal amino acids that are important for AmpD structure and/or function. Conservative substitutions, such as Ala94Val or Asp164Glu, highlight the specific steric and/or chemical requirements of the wild-type amino acids. For example, the intolerance for Ala94Val suggests that the smaller size of alanine is important here, because both amino acids are similarly hydrophobic. The Asp164Glu substitution involves similar charges, suggesting that this amino acid position makes important catalytic or structural contacts disrupted by the larger glutamic acid side chain. Asp164 is probably not simply a surface amino acid, because it has a seemingly stringent size requirement and because alanine at this position is also not tolerated.

Alignments show that *ampD* is highly conserved among various bacteria (e.g., Fig. 2). Asp164, Ala94, and the other substituted amino acids from our mutation studies are highly conserved among the aligned *ampD* sequences (Fig. 2) and further highlight their potential structural and/or functional importance.

The variety of loss-of-function mutations observed in this system suggests its utility for studying many kinds of mutation

mechanisms. This system may be useful additionally for studying the forward mutation spectra caused by potential damaging agents and environmental factors, because mutations in the small *ampD* gene are easily selected and sequenced.

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